

# Progenesis LC-MS Fractionation User Guide

Analysis workflow guidelines

for version 4.0



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#### Introduction

This user guide takes you through the processes involved in the analysis of a fractionated label free LC-MS experiment. Where the method of fractionation can be gel based on electrophoresis: 1D, 2D, Off gel etc or chromatography using an additional LC step prior to the LC-MS.

In this example, to demonstrate the processes at each stage, an experiment using samples that have been fractionated using ion exchange chromatography is described. As each fraction constitutes the full application of the label free workflow as described in the main LC-MS user guide a shortened version of these analysis steps are described here.

It starts with LC-MS data file loading then Alignment, followed by Analysis that creates a list of interesting features (peptides) which can be explored within Progenesis Stats using multivariate statistical methods, then onto Protein identity.

#### How to use this document

This document is designed to be used as a guide to the processes involved in the analysis of fractionated samples. Currently a full data set is not provided as this would result in a considerable download. The initial section of the document is concerned with an abbreviated description of the main experimental workflow as applied to a single fraction (a more comprehensive description of the main analysis workflow is available in the main User guide). The second section describes the process of recombining these individual fraction experiments into a 'Multi-fraction' experiment.

#### How can I analyse my own runs using LC-MS?

You can freely explore the quality of your LC-MS data using Data Import and then licence your own LC-MS runs using this evaluation copy of Progenesis LC-MS. Instructions on how to do this are included in a section at the end of the user guide document. Alternatively if you would like to arrange a demonstration in your own laboratory contact <u>support@nonlinear.com</u> and we will help you.

### LC-MS Data used in this user guide

NLD would like to thank Dr Robert Parker and Prof Haroun Shah at the Health Protection Agency, London, UK for providing the example data used in this user guide as well as invaluable discussion on the handling of the data.

# Workflow approach to LC-MS run analysis

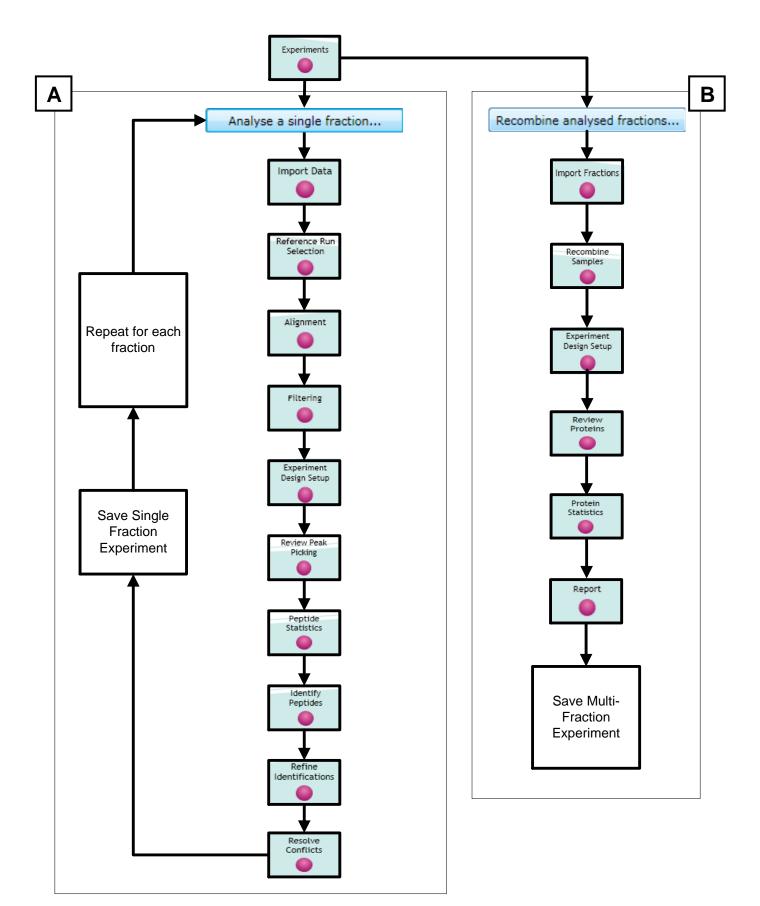
Progenesis LC-MS adopts an intuitive **Workflow** approach to performing comparative LC-MS data analysis. The following user guide describes the various stages of this workflow (see below) focusing mainly on the analysis of fractionated samples.

	Reference Run			Experiment						
Data Import	Selection	Alignment	Filtering	Design Setup	View Results	Progenesis Stats	Peptide Search	Peptide Filter	Protein View	Report

Stage	Description	Page
Import Data	<b>LC-MS Import Data:</b> Selection and review of data files for single fraction analysis.	7
Reference Run Selection	Reference Run Selection: Select run to align to.	7
Alignment	Alignment: automatic and manual run alignment	8
Filtering	<b>Filtering</b> : defining filters for features based on Retention Time, m/z , Charge and Number of Isotopes.	10
Experiment Design Setup	<b>Experiment Design Setup</b> : defining one or more group set ups for analysed aligned runs	11
Review Peak Picking	<b>Review Peak Picking</b> : review and validate results, edit peak detection, tag groups of features and select features for further analysis	12
Identify Peptides	<b>Identify Peptides</b> : managing export of MS/MS spectra to, and import of peptide ids from Peptide Search engines	13
Refine Identifications	Refine Identifications: manage peptide ids and filters	15
Resolve Conflicts	<b>Resolve Conflicts</b> : validation and resolution of peptide id conflicts for data entered from Database Search engines	16
Import Fractions	Import fractions: import multiple analysed fractionated experiments	20
Recombine Samples	Recombine samples: regenerate samples from fractions	22
Experiment Design Setup	Experiment design Setup: define original experimental design	24
Review Proteins	Review Proteins: review protein and peptide expression and identity	25
Protein Statistics	Protein Statistics: perform multivariate statistics on Proteins	27
Report	Report: generate reports on proteins of interest	28

### **Overview of a typical Fractionation Analysis workflow**

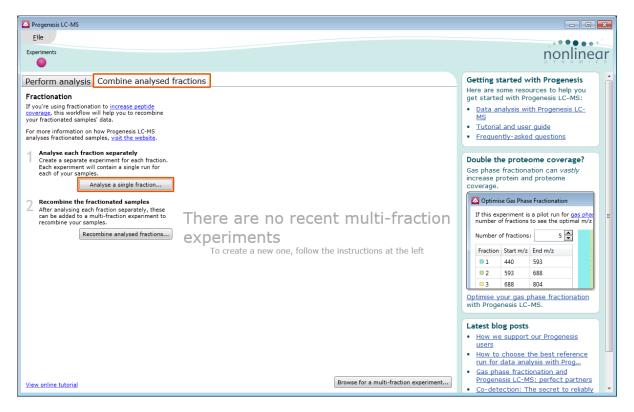
The workflow outlines the steps performed in the analysis of a typical fractionated experiment. In this example there were 6 fractions hence the main analysis workflow was performed 6 times. To analyse the data, select the **Combine analysed fractions** tab then (A) Analyse a single fraction then (B) Recombine the analysed fractions into a multi-fraction experiment.



### Analysing a single fraction

Open Progenesis LC-MS and click on the **Combine analysed fractions** tab to start the processing of Fractionated Samples.

To start the analysis of the LC-MS runs for a fraction, click on Analyse a single fraction...



This opens the 'Create New Experiment' dialog.

Name the fraction to be analysed (**Fract\_1**) then adjust the Data and Machine types accordingly and set the Experiment folder as required .

🔼 Create New Loms Experiment 🧾	
Create a new label-free experiment named:	
Fract_1	
Data type	
Profile data	
Centroided data	
Resolution (full width at half maximum) 50000	
Machine type	
High resolution mass spectrometer 🔹	
Experiment folder	
Save experiment in the same folder as the run data	
Choose an experiment folder	
Browse	
Browse	

Note: current settings shown are the defaults

Click Create experiment

# Stage 1: Data import and QC review of LC-MS data set for a fraction

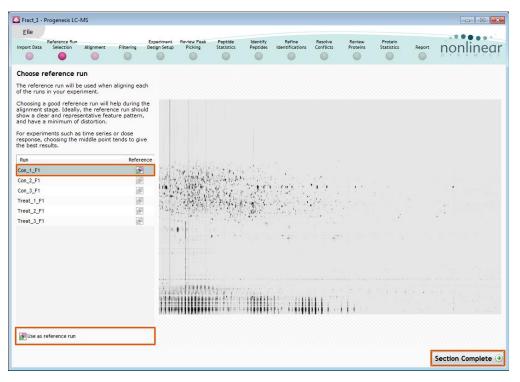
All LC-MS runs for the first Fraction are loaded into a new LC-MS experiment.

	ogenesis LC-N	ЛS											
Eile mport Data	Reference Run Selection	Alignment	Filtering	Experiment Design Setup		Peptide Statistics	Identify Peptides	Refine Identifications	Resolve Conflicts	Review Proteins	Protein Statistics	Report	nonlined
mport Dat	ta			Con	1_F1			-	<< Actio	ns •  >>			🕑 Hel
1 Import Select o click the Format: @ Abc Review Look at	your run da ne of the ava Import butto mzXML file out this data f the chroma all of the runs	ilable data fo on: s - format   + D tography s in the list b	Import	29 P	500	n i	1000		1500	m/z •	Peak     Tota	MS count: 8 count: 1,1	09,644 ty: 2.188e+009
might af think ha st style: # Co Im	ffect analysis. we significant Ion intensit n_1_F1 ported succes	Remove and problems. <u>n</u> y maps	tography that y runs that yo nore >> Import details	u j				l Macrice V	t muştarı Alanış				
Wis Im	n_2_F1 ported succes n_3_F1	ssfully		-			i if Pircip			<b>1</b> 27			
Tre	eat_1_F1 nding import.			60		el ce E		ing no					
	eat_2_F1 nding import.			80									
	eat_3_F1 nding import.												
				4 100 Retention Time (mins)									
Runs in t     Feature	the experiment detection: De cessing: Prof	fault		<ul> <li>Retention</li> </ul>					Zoom:				Section Complete

Once all the files have been imported move to the next stage in the workflow by clicking Section Complete.

#### Stage 2: Reference Run selection

This stage in the analysis workflow allows you to review and select the most appropriate Reference LC-MS run to align all the other runs to.



To select a Reference run either click on the run in the list and then click **Use as reference run** or double click on the run in the list.

Now move to the next stage in the workflow by clicking Section Complete.

# Stage 3: Alignment

At this stage Progenesis LC-MS Alignment opens displaying your data.



#### Generation of alignment vectors

The alignment of LC-MS runs is required in the LC (retention time) direction, this is key to correcting for the variable elution of peptides during the chromatographic separation.

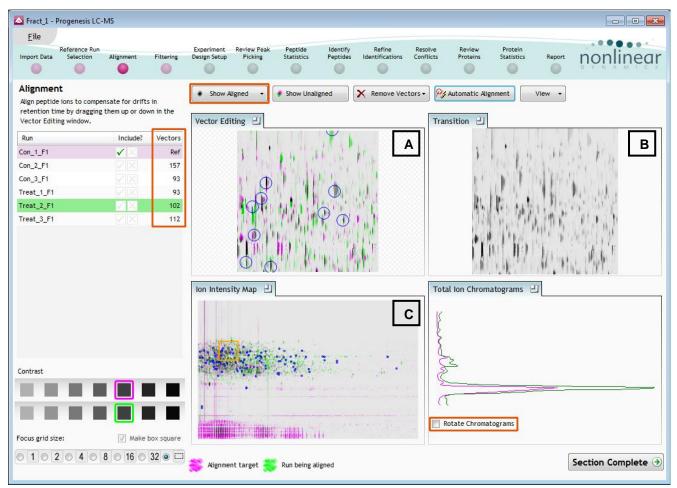
The alignment vectors are generated automatically for all the LC-MS runs by using the 'Automatic vector wizard' accessed by clicking on **Automatic Alignment** on the top tool bar, and making sure all the runs are selected before clicking OK.

Resolv Conflic			
ctors 🕶	𝒫 Automatio	c Alignment	Vi
	Transition	<u>-</u> ]	

#### **Reviewing generation of alignment vectors**

After applying **Automatic alignment** the number of vectors will be updated on the **Runs** panel and the vectors will appear (in blue) on the view.

If the alignment has worked well then in Windows A and C the grid lines should show minimal distortion, Window B (Transition) will show features pulsing slightly but not moving up and down.



At this point, you should check the automatically placed (blue) vectors. This will be easier with a larger grid size. Make sure the grid size is set to 4 using the '**Focus grid size**' control at the bottom left of the window.

In each square, you can, if required edit the vectors to improve the run alignment (for more detailed information on performing the alignment of your runs refer **Appendix 1** (page 32) and also to the main LC-MS analysis User guide.

### Stage 4: Filtering

Now that you have reviewed your aligned Runs, you are ready to analyse them. Move to the **Filtering** stage, by either clicking on **Section Complete** (bottom right) or on Filtering on the workflow.



#### **Peak Picking Parameters**

The Peak Picking Parameters dialog opens, showing all the runs in the current experiment and a tick against each run. This is the default setting, where the peak picking algorithm uses information from all of the runs to contribute to the pattern of feature outlines.

🔼 Peak Picking Parameters		Peak Picking Parameters	
Runs for peak picking Peak picking lim	its Maximum charge	Runs for peak picking Peak picking I	imits Maximum charge
Choose runs for peak picking		Sensitivity	
You can tick or un-tick each run to control which will be used by the peak picking algorithm. Although any run which is left un-ticked will not affect the feature outlines, it will still have outlines added to it and will be available in the experiment design setup. Learn more about why you might not want to select all runs.	<pre>✓ Con_1_F1 ✓ Con_2_F1 ✓ Con_3_F1 ✓ Treat_1_F1 ✓ Treat_2_F1 ✓ Treat_3_F1</pre>	You can adjust the sensitivity of the peak picking algorithm using these different methods. Each sensitivity method examines the intensities of groups of MS peaks to judge whether they are likely to form part of an ion or whether they represent noise and so should be ignored. Peaks that are rejected as noise will not be used to build ion outlines.	Absolute ion intensity     % Base Peak      The automatic sensitivity method uses a noise estimation algorithm to determine the sonsitivity value, the more features will be detected.      fewer default more
		Minimum retention time win The retention time window is the period over which an ion has eluted. If you set a retention time window limit, any ion that has eluted over a shorter period will be rejected.	dow Apply a retention time window limit RT window limit: minutes
	Start peak picking Cancel		Start peak picking Cancel

*Tip*: It may be appropriate **only** to pick peaks that are present in a limited number of your runs. In which case un-tick the runs that you do **NOT** want to contribute to the feature detection pattern. This may be important when one or more of the runs appear noisy due to non-optimal chromatography or sample handling.

**Note**: feature outlines will be added to 'un-ticked' runs; although these runs will not contribute to the peak picking pattern.

*Tip*: depending on run quality, a suggested minimum number of ticked runs should include at least one replicate of each experimental condition.

The sensitivity of the detection can be controlled by adjusting settings under the Peak picking limits tab.

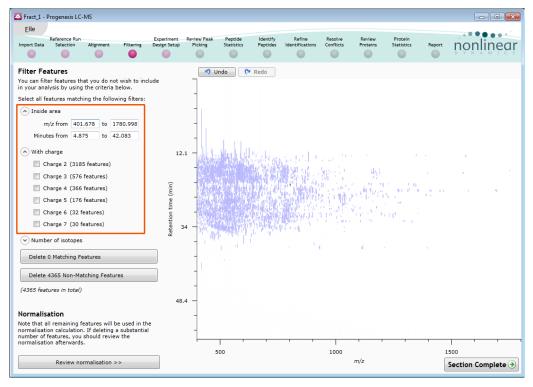
For this example the default settings for the <b>Automatic</b> method were used.	Runs for peak picking Parameters
The third tab allows you to set the <b>maximum charge</b> of the ions that will be detected. The default setting is a charge state of 20.	Maximum allowable charge You can set the maximum charge of ions to be detected. Ions with a charge greater than this value will be rejected.
Press Start Peak Picking to start the detection process.	
During the few minutes that the automatic analysis requires, a progress bar will appear telling you that it is Analysing.	
Analysing	

Peak Picking Paramete	rs		
Runs for peak picking	Peak picking limits	Maximum charge	
Maximum allowat	ole charge		
You can set the maxir of ions to be detected charge greater than t be rejected.	. Ions with a	Maximum ion charge:	20
		Start peak	picking Cancel

More details on the management of sensitivity are available in the How to do on Adjusting the Sensitivity of Feature detection.

On completion of analysis the Filtering stage will open displaying the number of features.

If required you can remove features based on position, charge state, number of isotopes or combinations of these feature properties.



The example here shows the removal of features with a charge state of **less than 2 and greater than 7** and outside a defined retention time window.

Having removed features the Normalisation will recalculate as you move to the next section.

#### Stage 5: Experiment Design Setup for Analysed Runs

At this stage in the workflow you can setup one or more experimental designs for your data.

There are two basic types of experimental designs:

Between-subject design: where samples from any given subject appear in only one condition.

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ring De		Review Peak Picking	Peptide Statistics	Identify Peptides	Refine Identifications	Resolve Conflicts	Review Proteins			port n	onli	near
												🕜 Help 🔻
do you '	want to	use for t	his experi	ment?								
gn				C	HO Within	-subject	Design					*
A		maria LC MS	Delete				ent		Before	During	After	
			experin	nent de	sign			Patient X	X1	X2	X3	E
c		Control						Patient Y	Y1	¥2	¥3	
Add cone	· ·	oy layout f	rom:	1			-	Patient Z	Z1	Z2	Z3	
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Within-subject design: where samples have been taken from a given subject under different conditions

(Additional information on how to apply the Within-subject Design is in Appendix 2 page 37)

This experiment contains 2 conditions: Control and Treated and uses the **Between-subject design** to group the analysed runs to reflect the Biological conditions in the original study.

Select Between-subject and give design an appropriate name.

Highlight the runs, to add them on to a new condition by clicking on Add Selected Runs to Condition

Fract_1 - Progenesis LC-MS										
Eile Import Data Reference Run Selection Alignmer	nt Filtering Des	oeriment Review F ign Setup Pickin	Peak Peptide g Statistics	Identify Peptides	Refine Identifications	Resolve Conflicts	Review Proteins	Protein Statistics	Report	nonlinear
Control vs Treated I	× 🗅 New									🕜 Help 🔻
Setup conditions			Add Selected R	uns to Con	dition 👻 S	earch		Q		
Setup the conditions that you w (e.g., control, drug A, etc), and your samples to the correct cor	then assign each		Add to new	condition		Treat_	_2F1		Tr	eat_3_F1
Control	Dele Con_1_F1 Remov		Control							
	Con_3_F1 Remo	<u>/e</u>								
Add condition										
									2	Section Complete 🏵

Click Section Complete to move to View Results.

#### Stage 6: Validation, review and editing of results

The purpose of this stage in the Workflow is to review the list of features using the visual tools provided and edit features if required.

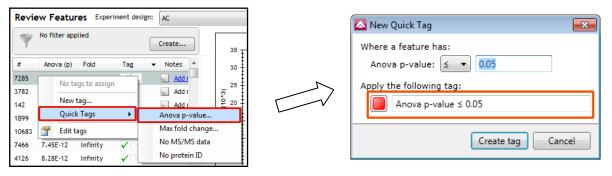
The review stage has 4 display modes: 1D, 2D, 3D and Feature Details controlled by the tabs on the bottom left of the display and the expander bar to the right of the table. Each display has multiple views to allow comparative exploration of the detected features on the aligned LC-MS runs.



Details on how to use the various views and table are described in detail in the Main analysis workflow userguide.

For the purposes of this example we require to identify all those features that demonstrate a significant Anova value ( $p \le 0.05$ ) between the 2 conditions being studied. We will create a Tag identifying just those features.

Right click on a feature in the table and select **Quick Tags** then **Anova p-value**.. set the required threshold and either accept the tag name or overtype it.

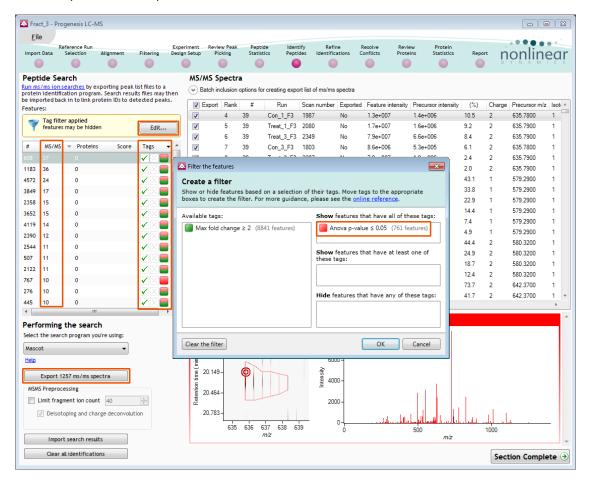


Now move to the Peptide Search stage in the workflow using the icon on the workflow.

#### **Stage 7: Identify Peptides**

Progenesis LC-MS does not perform peptide identifications itself. Instead it supports identifications by allowing you to export a set of MS/MS peak lists in formats which can be used to perform peptide searches by various search engines. The resulting identifications can then be imported back into Progenesis LC-MS, using a number of different file types, and matched to your detected features.

Determining protein identification is dependant on the availability of MS/MS data for the LC-MS runs. This data may be available but limited if the LC-MS was performed in a data dependant MS/MS detection mode due to under sampling. Under these conditions MS/MS data acquisition is dependant on thresholds and parameters set prior to the acquisition of the LC-MS run.



For this example we are using LC-MS runs containing MS/MS data where the data was acquired in a data dependant mode.

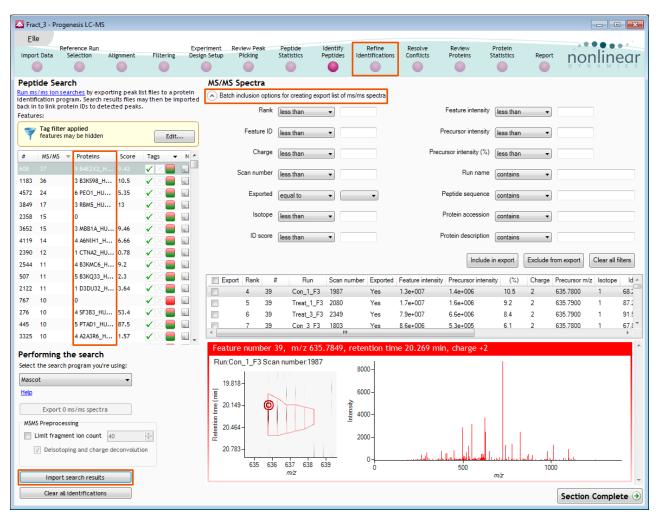
The Peptide Search page shows the number MS/MS that have been matched to each feature in the Feature list (see above). MS/MS scans are matched to a feature if their precursor m/z and aligned retention time fall within the area of one of the isotopes of the feature. The MS/MS scans which are matched to the displayed features are shown in the MS/MS spectra list on the right.

The first step is to decide which MS/MS scans you wish to export to be identified. By default this is all the available spectra for the Features displayed in the Features list. Using the tag created in the previous section you can filter the table to only those showing a significant change (Anova

 $p \le 0.05$ ) between the conditions. This number of spectra to be exported is visible on the Export button.

#### Performing an MS/MS Ion Search

- 1. Select appropriate search engine i.e. Mascot
- 2. Click 'Export current query set' to save search as file
- 3. Perform search on appropriate search engine and save results file
- 4. Click 'Import search results', locate results file and open
- 5. On importing the Search results the Features table updates to reflect the identified proteins and the relevant score for each searched feature.



In order to review, and refine the quality of the **Search** results click on the next stage in the workflow, **Refine Identifications**.

Performing the search									
Select the search program you're using:									
Mascot 👻									
Help									
Export 0 ms/ms spectra									
MSMS Preprocessing									
Limit fragment ion count 40									
✓ Deisotoping and charge deconvolution									
Import search results									
Clear all identifications									

### **Stage 8: Refine Identifications**

In this example the organism under study is Clostridium difficile

As an **example** 'Acceptance Criteria' on which to base the sequential filtering of the Peptide results, the following thresholds will be applied:

- Remove identifications with a Score less than 30
- Remove identifications where less than 2 hits were returned
- Remove all identifications where the Protein Description Contains 'hypothetical'
- Remove all identifications where the Protein Description Doesn't contain 'Clostridium difficile'

On the Batch detection options panel, set the Score to less than 30, then Delete matching search results.

🔼 Frac	t_3 - Proge	nesis LC-I	MS															
Eile																		
_		erence Ru			Filhering	Experin		Review Pea		tide	Ident		Refine	Resolve	Review	Protein	Deserve	
Import	Data S	election	Aligni	ment	Filtering	Design S	etup	Picking		istics	Pepti		lentification		Proteins	Statistics	Report	nonlinear
					-								•					
Featu	ires				Pep	tide Sea	rch R	esults										
#	Total Hits	m/z		Charge	Bat	ch deletion	options											
1	33 19	554.82	27.12	2		1	icore (	less than	•	30				Chi	arge less than	•		
4	29	622.85 596.32	24.03 27.89	2						_								
5	14	702.38	28.85	2			Hits	less than	•					Seque	ence contains	•		
6	7	523.78	20.08	2														
7	22	412.22	18.81	2			Mass	less than	•					Acces	contains	•		
8	36 9	694.91	33.98	2		Mass error (	nnm) (	less than				_		Descrip	tion contains	•		
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17	12 9	663.86 544.76	29.96 17.47	2										_				
20	9 14	751.33	20.68	2	-	Sequence Le	ength (	less than	•					De	lete matching se	arch results	Delete n	on-matching search results Clear all filters
22	27	588.32	29.60	2													·	
23	9	647.82	17.82	2	-								1 1					•
24	57	679.35	27.78	2		#	Score	Hits	m/z		) Charge	Mass	Mass erro	Sequence	Accession		fications	*
25	8	516.30	26.87	2			56.89	16	554.82	27.12	2	1107.63		VPLVAPEDLR	A8MZ91_HU			(A8MZ91) Protein arginine methyltransfera
26	14 27	624.29 487.72	20.15 18.33	2			3.25	1	554.82	27.12	2	1107.63	85.20		PRS7_HUM		dation (M)	(P35998) 265 protease regulatory subunit 7
28	110	744.35	26.44	2			56.89	Delete	5463 searc	h result	s?					83		(B2RDD7) cDNA, FLJ96564, highly similar to
31	14	548.23	19.22	2			15.43						_					(Q9HDC9) Adipocyte plasma membrane-ass
32	9	603.81	20.63	2			43.05	?	Are yo	u sure y	ou want	to perma	anently del	ete 5463 peptide s	earch results?			(Q9BQA1) Methylosome protein 50
33	3	607.75	23.42	2			15.43	-										(A2A2F9) Chromosome 20 open reading fra
34 35	4	823.43 495.72	18.81 17.63	1			41.40										dation (M)	(Q99460) 265 proteasome non-ATPase regu
36	72	723.85	23.38	2			41.40							Yes	No		dation (M)	(B2R6D0) cDNA, FLJ92896, highly similar to
38	4	755.36	25.78	2			5.16							res	INO			(Q5W0B1) RING finger protein 219
39	18	635.78	20.27	2			100.27		502.30	20.05		1902-19	45.50					(Q9BQA1) Methylosome protein 50
40	18	603.33	28.07	2			13.90	4	523.78	20.08	2	1045.54		VERVTISSR	H90B4_HU/			(Q58FF6) Putative heat shock protein HSP (
41	4 23	679.51 617.31	28.44 24.06	1			5.61 9.75	1	523.78 523.78	20.08	2	1045.54			SMG1_HUM			(Q96Q15) Serine/threonine-protein kinase (O95785) Protein Wiz
43	1	697.38	32.41	2				2						ITITGYR	NOMO1_HL			
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45	1	789.41	28.30	2			10.37 21.72	8	412.22	18.81	2	822.42	44.98 5.70	LTEWFK	GCN1L_HU			(Q9H049) Putative uncharacterized protein (Q92616) Translational activator GCN1
46	9 7	606.34	28.41	2	<b>V</b>		21.72 5.87	8	412.22 694.91	33.98	2		5.70		-		dation (M)	(Q92616) Translational activator GUN1 (B3KS15) cDNA FLJ35278 fis, clone PROST2
48	1	776.41 604.32	25.74 28.59	2		0		0	074.71	33.70	2				n	and, [1] OXI		(BARGED) CONNET EUGDZZO TIS, CIONE PROSTZ
50	12	761.89	25.32	2	-					_			"					•
52 4	<b>?</b> ?	REE 91	74 04	• ·	7422	search resu	lts. 546	3 matching	batch del	ete optio	ons.							Section Complete 🏵

**Note**: the search results matching the filter criteria turn pink and the number of search results being deleted is displayed

Now Clear all filters and then apply the next filters as described above.

To validate the Peptide search results at the protein level select the next stage in the workflow by clicking on **Protein View**.

### Stage 9: Resolve Conflicts

The Protein View combines the quantitative LC-MS data with the qualitative MS/MS results at the protein level, highlighting proteins of interest between experimental groups. This stage allows you to examine the behaviour of the identified peptides and resolve any conflicts for the various peptide assignments at the protein level.

The Resolve Conflicts view provides a number of interrelated graphical and tabular views to assist you in the validation of the peptides that have been assigned to proteins and also to review the relevance of the data returned from the search.

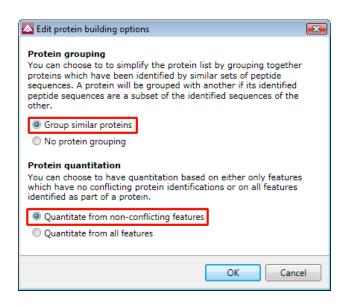
Open the Resolve Conflicts and order the data in the Proteins table (A) on the basis of **Conflicts**. Note: the look of the tables (with regards to ordering) in the following section may vary slightly.

Proteins	V N	o filter applied	Create		pride	es of gi	10009	37					No filter app	lied	Create
Accession Pe	ptides Cor	flicts Score	Anova (p)*	*	#	Score	Hi	ts Mass	Mass error (p	RT (mins)	Charge	Tags	✓ Abundance	Confli	cts Peptide
🌛 gi 5668937 🛛 🔾	12 1	4 1.41E+03	2.03E-05 3	-	446	6 93.3	31	0 1676.838	1.2	34.5	2	$\checkmark$ $\times$	7.47E+05	1	🌒 IRD
🎯 gi 126697810	9 1	4 1.13E+03	4.55E-05 3	V	333	5 84.	7 4	4 1423.65	0.405	22.5	2	✓ ×	7.39E+04	1	S DTD
🗿 gi 209571234	24 1	3 2.4E+03	4.05E-05 2	V	147	7 101	1 1	0 1230.609	0.44	22.7	2	✓ ×	3.07E+06	1	🌖 AAD
🗿 gi 260682215	23 1	3 2.03E+03	6.28E-05	V	166	6 125	5 1	0 2317.115	0.168	38.7	1	B	5.55E+06	1	🌖 LES
🗿 gi 126698450	12	1.21E+03	4.05E-06 🕈	1	179	9 60.9	9 9	2317.115	0.201	38.7		P∕×	3.09E+06	1	🔇 LES
谢 gi   126700407	9	1.04E+03	0.945 1	1	238	8 107	7 1	0 1716.857	0.429	30.4	2	<ul><li>✓ ×</li></ul>	1.73E+06	1	🌖 VNT
🌍 gi 255656776	9	925	0.00888 1	V	564	4 51.3	2 4	1716.858	0.394	30.4	3	<b>√</b> ×	3.57E+05	1	🔇 VNT
gi   255654924	7	645	8.66E-06 1		283	3 49.	51	0 1676.838	1.32	34.5	3	<b>V</b> X	7.59E+05	1	П и п и п и п и п и п и п и п и п и п и
Protein: gi 566893 Protein: gi 126693		gellin subunit	[Clostridium d	difficile	e 630	]									
Protein: gi 12669 eptide Views Protein R Conflicting protein	lesolution ns for fea	iti <b>c</b> e •46		Pept	ides (	of gi 12					Channes 7		11	D	Deskide Ca
Protein: gi 12669 eptide Views Protein R Conflicting protein Accession Pep	esolution ns for fea ptides Co	itive 46	e Peptid	Pept	ides (	of gi 12 Score	Hits	Mass /		RT (mins)	-		Abundance 3.07F+06	D Conflict	Peptide See
Protein: gi 12669 eptide Views Protein R Conflicting protein Accession Pep gi 16668937 • 1	lesolution ns for fea	ture 46 ists Protein Score 1.41E+03		Pept	ides (	of gi 12			Aass error (p R 0.44 0.168	RT (mins) 22.7 38.7	2		<ul> <li>Abundance</li> <li>3.07E+06</li> <li>5.55E+06</li> </ul>	Conflict	S AADDI
Protein: gi 12669 eptide Views Protein R Conflicting protein Accession Pep @ gi 5668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept	ides ( r 147	of gi 12 Score 101	Hits 10	Mass /	0.44	22.7	2		3.07E+06	Conflict	
Protein: gi 12669 eptide Views Protein R Conflicting protein Accession Pep @ gi 5668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept	ides ( 147 166	of gi   12 Score 101 125	Hits 10 10	Mass / 1230.609 2317.115	0.44	22.7 38.7	2 v 2 v		3.07E+06 5.55E+06	Conflict	S AADDI LEST
Protein: gi 12669 eptide Views Protein R Conflicting protein Accession Pep @ gi 5668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept #	ides ( 147 166 179	of gi   12 Score 101 125 60.9	Hits 10 10 9	Mass / 1230.609 2317.115 2317.115	0.44 0.168 0.201	22.7 38.7 38.7	2 v 2 v 3 v		3.07E+06 5.55E+06 3.09E+06	Conflict	AADD: LEST( LEST(
Protein: gi 12669 sptide Views Protein R Conflicting protein Accession Pep gi 5668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept #	ides ( 147 166 179 238	of gi 12 Score 101 125 60.9 107	Hits 10 10 9 10	Mass // 1230.609 2317.115 2317.115 1716.857	0.44 0.168 0.201 0.429	22.7 38.7 38.7 30.4	2 v 2 v 3 v 2 v		3.07E+06 5.55E+06 3.09E+06 1.73E+06	Conflict	<ul> <li>AADD;</li> <li>LEST;</li> <li>LEST;</li> <li>VNTN;</li> </ul>
Protein: gi 12669 sptide Views Protein R Conflicting protein Accession Pep gi 5668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept # # V V V V V V V V V V V V V V V V V	ides ( 147 166 179 238 564	of gi   12 <u>Score</u> 101 125 60.9 107 51.2	Hits 10 10 9 10 4	Mass         J           1230.609         2317.115           2317.115         1716.857           1716.858         1716.858	0.44 0.168 0.201 0.429 0.394	22.7 38.7 38.7 30.4 30.4	2 2 3 2 3 3 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05	Conflicts 1 1 1 1 1	<ul> <li>AADD;</li> <li>LEST;</li> <li>LEST;</li> <li>VNTN;</li> <li>VNTN;</li> </ul>
Protein: gi 12669 sptide Views Protein R Conflicting protein Accession Pep gi 5668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept # # V V V V V V V V V V V V V V V V V	ides ( 147 166 179 238 564 283	of gi   12 Score 101 125 60.9 107 51.2 49.5	Hits 10 10 9 10 4 10	Mass         J           1230.609         2317.115           2317.115         1716.857           1716.858         1676.838	0.44 0.168 0.201 0.429 0.394 1.32	22.7 38.7 38.7 30.4 30.4 34.5	2 2 3 2 3 3 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05	Conflict 1 1 1 1 1 1 1	<ul> <li>AADD:</li> <li>LEST(</li> <li>LEST(</li> <li>VNTN'</li> <li>VNTN'</li> <li>IRDTI</li> </ul>
Protein: gi 12669 eptide Views Protein R Conflicting protein Accession Pep @ gi 5668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept	ides ( 147 166 179 238 564 283 446	of gi   12 Score 101 125 60.9 107 51.2 49.5 93.3	Hits 10 10 9 10 4 10 10	Mass         J           1230.609         2317.115           2317.115         1716.857           1716.857         1716.858           1676.838         1676.838	0.44 0.168 0.201 0.429 0.394 1.32 1.2	22.7 38.7 38.7 30.4 30.4 34.5 34.5	2 v 2 v 3 v 2 v 3 v 3 v 2 v 3 v 2 v 2 v 2 v		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05	Genflict 1 1 1 1 1 1 1 1	<ul> <li>AADD:</li> <li>LEST(</li> <li>LEST(</li> <li>VNTN'</li> <li>VNTN'</li> <li>IRDTI</li> <li>IRDTI</li> </ul>
Protein: gi 12669 eptide Views Protein R Conflicting protein Accession Pep gi 15668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept  #  #  #  #  #  #  #  #  #  #  #  #  #	ides ( 147 166 179 238 564 283 446 431	of gi   12 Score 101 125 60.9 107 51.2 49.5 93.3 49.6	Hits 10 9 10 4 10 10 8	Mass         J           1230.609         2317.115           2317.115         1           1716.857         1           1716.858         1           1676.838         1           1692.835         1	0.44 0.168 0.201 0.429 0.394 1.32 1.2 0.206	22.7 38.7 30.4 30.4 34.5 34.5 20.6	2 v 2 v 3 v 2 v 3 v 3 v 3 v 3 v 3 v 3 v 3 v 3 v		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05	Genflict 1 1 1 1 1 1 1 1	<ul> <li>AADD:</li> <li>LEST:</li> <li>LEST:</li> <li>VNTN'</li> <li>VNTN'</li> <li>IRDTI</li> <li>IRDTI</li> <li>IRDTI</li> </ul>
Protein: gi 12669 eptide Views Protein R Conflicting protein Accession Pep @ gi 5668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept	ides ( 147 166 179 238 564 283 446 431 789	of gi   12 Score 101 125 60.9 107 51.2 49.5 93.3 49.6 103	Hits 10 9 10 4 10 10 8 10	Mass         J           1230.609         2317.115           2317.115         1           1716.857         1           1716.858         1           1676.838         1           1692.835         1           1692.833         1	0.44 0.168 0.201 0.429 0.394 1.32 1.2 0.206 1.09	22.7 38.7 30.4 30.4 34.5 34.5 20.6 20.6	2 v 2 v 3 v 2 v 3 v 3 v 3 v 2 v 3 v 2 v 2 v 2 v		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05 4.62E+05	Genflict 1 1 1 1 1 1 1 1	<ul> <li>AADD;</li> <li>LEST;</li> <li>LEST;</li> <li>VNTN;</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> </ul>
Protein: gi 12669 eptide Views Protein R Conflicting protein Accession Pep gi 15668937 • 1	Resolution Ins for fea Dides Cont 12 14	ture 46 ists Protein Score 1.41E+03	e Peptid	Pept # # # # # # # # # # # # # # # # # # #	ides ( 147 166 179 238 564 283 446 431 789 525	of gi   12 Score 101 125 60.9 107 51.2 49.5 93.3 49.6 103 104	Hits 10 10 9 10 4 10 10 8 10 10	Mass         J           1230.609         2317.115           2317.115         1           1716.857         1           1716.858         1           1676.838         1           1692.835         1           1692.833         1	0.44 0.168 0.201 0.429 0.394 1.32 1.2 0.206 1.09 0.139	22.7 38.7 38.7 30.4 30.4 34.5 34.5 20.6 20.6 36	2 v 2 v 3 v 2 v 3 v 3 v 2 v 3 v 2 v 2 v 2 v		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05 4.62E+05 5.46E+05	Genflict 1 1 1 1 1 1 1 1	<ul> <li>AADD;</li> <li>LEST;</li> <li>LEST;</li> <li>VNTN;</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> <li>VNTN;</li> </ul>

In the above example the conflict would be resolved in favour of the protein with 12 peptides as the flagellin subunit does not contain any unique peptides as compared to flagellin.

**Note**: the number of conflicts you have to resolve will depend on the scope and stringency of the filters you apply at the **Peptide Search** stage,

**Note**: with the default **Protein options** for protein grouping and Protein quantitation are set as shown.



Having performed the conflict resolution with **Group similar proteins** and **Quantitate from non-conflicting features** now switch off the protein grouping.

As grouping is switched off the grouped proteins appear with conflicts to the other group members and the number of unique peptides that are used for quantitation appear in brackets after the peptide number.

	9	No filt	er applied	Create	•	Рер	lides	of gi 566	0937					No filter ap	plied	Create
Accession	Peptides	Conflict	Score	Anova (p)*	*		#	Score	Hits Mas	Mass error (p	. RT (mins)	Charge	Tags	✓ Abundance	Confli	icts Peptide 9
) gi   5668937	12 (3)	14	1.41E+03	4.15E-06	3	<b>V</b>	446	93.3	10 1676	.838 1.2	34.5	2	$\checkmark$ ×	7.47E+05	1	🕥 IRD
🕽 gi   126697810	9 (0)	14	1.13E+03		1	V	3335	84.7	4 142	3.65 0.405	22.5	2	$\checkmark$ ×	7.39E+04	1	S DTE
gi 209571234	24 (12)	13	2.4E+03	2.51E-07	e	V	147	101	10 1230	0.609 0.44	22.7	2	<ul><li>✓ ×</li></ul>	3.07E+06	1	S AAD
🕽 gi 260682215	23 (11)	13	2.03E+03	4.08E-05	3	1	166	125	10 2317	.115 0.168	38.7	2	<b>√</b> ×	5.55E+06	1	🔇 LES
🕽 gi 126698450	12 (5)	9	1.21E+03	1.28E-06	2	1	179	60.9	9 2317	.115 0.201	38.7	3	<ul><li>✓ ×</li></ul>	3.09E+06	1	🔇 LES
gi   126700407	9 (2)	9	1.04E+03	0.000764	3 +	1	238	107	10 1716	.857 0.429	30.4	2	<ul><li>✓ ×</li></ul>	1.73E+06	1	S VN 2
onflicting pro	oteins toi	r teatur	e 446			entir	les ot	01117664	0/810							
Conflicting pro			e 446 Protein Score	Pept	id	#		gi 12669 pre Hi		Mass error (p	RT (mins)	Charge 1	Fags	<ul> <li>Abundance</li> </ul>	Conflicts	Peptide Ser
Accession	Peptides 12 (3)	Conflicts	Protein Score 1.41E+03	<b>93.3</b>	id [	# / 14	Sco 7	ore Hi 101 1	ts Mass 0 1230.60	9 0.44	22.7	2 🗸		3.07E+06	Conflicts	
	Peptides	Conflicts	Protein Score		id [	# 7 14 7 16	5c	ore Hi 101 1 125 1	ts Mass 0 1230.60 0 2317.11	9 0.44 5 0.168	22.7 38.7	2 .		3.07E+06 5.55E+06		Peptide Ser
Accession	Peptides 12 (3)	Conflicts	Protein Score 1.41E+03	<b>93.3</b>	id	# Z 14 Z 16 Z 15	5c	Dre Hi 101 1 125 1 50.9 9	Mass           0         1230.60           0         2317.11           0         2317.11	9 0.44 5 0.168 5 0.201	22.7 38.7 38.7	2 2 3		3.07E+06 5.55E+06 3.09E+06	1 1 1	<ul> <li>AADD;</li> <li>LEST(</li> <li>LEST(</li> </ul>
Accession	Peptides 12 (3)	Conflicts	Protein Score 1.41E+03	<b>93.3</b>	id E	# 7 14 7 16 7 17 7 23	5c 17 16 19	Dre         Hill           101         1           125         1           50.9         5           107         1	Mass         Mass           0         1230.60           0         2317.11           0         2317.11           0         1716.85	9 0.44 5 0.168 5 0.201 7 0.429	22.7 38.7 38.7 30.4	2 2 3 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06	1 1 1 1	<ul> <li>AADD;</li> <li>Lest;</li> <li>Lest;</li> <li>VNTN;</li> </ul>
Accession	Peptides 12 (3)	Conflicts	Protein Score 1.41E+03	<b>93.3</b>	id I I I I I	# 7 14 7 16 7 17 7 23 7 56	500 17 16 19 18 18	Dre         Hill           101         1           125         1           50.9         5           107         1           51.2         4	Mass           0         1230.60           0         2317.11           0         2317.11           0         1716.85	9 0.44 5 0.168 5 0.201 7 0.429 8 0.394	22.7 38.7 38.7 30.4 30.4	2 2 3 2 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05	1 1 1	<ul> <li>AADD;</li> <li>LEST(</li> <li>LEST(</li> <li>VNTN'</li> <li>VNTN'</li> </ul>
Accession	Peptides 12 (3)	Conflicts	Protein Score 1.41E+03	<b>93.3</b>		# 7 14 7 16 7 17 7 23 7 56 7 24	5c 17 16 19 18 13	bre Hii 101 1 125 1 50.9 5 107 1 51.2 4 49.5 1	Mass           0         1230.60           0         2317.11           0         2317.11           0         1716.85           0         1716.85           0         1676.83	9 0.44 5 0.168 5 0.201 7 0.429 8 0.394 8 1.32	22.7 38.7 38.7 30.4 30.4 30.4 34.5	2 2 3 2 3 3 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05	1 1 1 1 1 1 1	<ul> <li>AADD;</li> <li>LEST;</li> <li>LEST;</li> <li>VNTN;</li> <li>VNTN;</li> <li>IRDT;</li> </ul>
Accession	Peptides 12 (3)	Conflicts	Protein Score 1.41E+03	<b>93.3</b>		# 7 14 7 16 7 17 7 23 7 56 7 24 7 44	500 17 19 18 13 13 16	Free         Hi           101         1           125         1           50.9         5           107         1           51.2         4           49.5         1           93.3         1	Mass           0         1230.60           0         2317.11           0         2317.11           0         1716.85           1         1716.85           0         1676.83           0         1676.83	9 0.44 5 0.168 5 0.201 7 0.429 8 0.394 8 1.32 8 1.2	22.7 38.7 30.4 30.4 30.4 34.5 34.5	2 2 3 2 3 3 3 2 2 3 2 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05	1 1 1 1	<ul> <li>AADD;</li> <li>LEST;</li> <li>LEST;</li> <li>VNTN'</li> <li>VNTN'</li> <li>IRDT;</li> <li>IRDT;</li> </ul>
Accession	Peptides 12 (3)	Conflicts	Protein Score 1.41E+03	<b>93.3</b>		# 7 14 7 16 7 17 7 23 7 56 7 24 7 44 7 43	Sco 17 16 19 18 18 13 13 14 14 11		Mass           0         1230.60           0         2317.11           0         2317.11           0         1716.85           4         1716.85           0         1676.83           0         1676.83           1         1692.83	9         0.44           5         0.168           5         0.201           7         0.429           8         0.394           8         1.32           8         1.2           5         0.206	22.7 38.7 38.7 30.4 30.4 30.4 34.5 34.5 20.6	2 2 3 2 3 3 2 3 2 3 3 2 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05	1 1 1 1 1 1 1	<ul> <li>AADD;</li> <li>LEST;</li> <li>LEST;</li> <li>VNTN'</li> <li>VNTN'</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> </ul>
Accession	Peptides 12 (3)	Conflicts	Protein Score 1.41E+03	<b>93.3</b>		# 7 14 7 16 7 17 7 23 7 24 7 24 7 44 7 43 7 74	5cc 17 16 19 18 14 13 13 16 11 19	Free         Hi           101         1           125         1           50.9         5           107         1           51.2         4           49.5         1           93.3         1	Mass           0         1230.60           0         2317.11           0         2317.11           0         1716.85           1         1716.85           0         1676.83           1         1692.83           0         1692.83	9         0.44           5         0.168           5         0.201           7         0.429           8         0.394           8         1.32           8         1.2           5         0.206           3         1.09	22.7 38.7 30.4 30.4 30.4 34.5 34.5	2 2 3 2 3 2 3 3 2 3 2 2 2 3 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05	1 1 1 1 1 1 1	<ul> <li>AADD;</li> <li>LEST;</li> <li>LEST;</li> <li>VNTN'</li> <li>VNTN'</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> </ul>
Accession gil5668937 gil126697810	Peptides 12 (3)	Conflicts	Protein Score 1.41E+03	<ul><li>✓ 93.3</li><li>✓ 93.3</li></ul>		# 2 14 2 16 2 17 2 25 2 28 2 28 2 44 2 43 2 78 2 53	5cc 17 16 19 18 14 13 13 16 11 19	International         International           101         1           101         1           125         1           125         1           107         1           51.2         4           49.5         1           93.3         1           103         1	Mass           0         1230.60           0         2317.11           0         2317.11           0         1716.85           1         1716.85           0         1676.83           1         1692.83           0         1692.83	9         0.44           5         0.168           5         0.201           7         0.429           8         0.394           8         1.32           8         1.2           5         0.206           3         1.09	22.7 38.7 38.7 30.4 30.4 34.5 34.5 20.6 20.6	2 2 3 2 3 3 2 3 2 3 3 2 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05 4.62E+05	1 1 1 1 1 1 1	<ul> <li>AADD;</li> <li>LEST;</li> <li>LEST;</li> <li>VNTN'</li> <li>VNTN'</li> <li>IRDT;</li> <li>IRDT;</li> <li>IRDT;</li> </ul>

With protein grouping switched on protein groups and the additional members are indicated by a bracketed number located after the Accession number. Taking **flagellin** as an example, when the cursor is held over the accession number the group members appear in a tool tip.

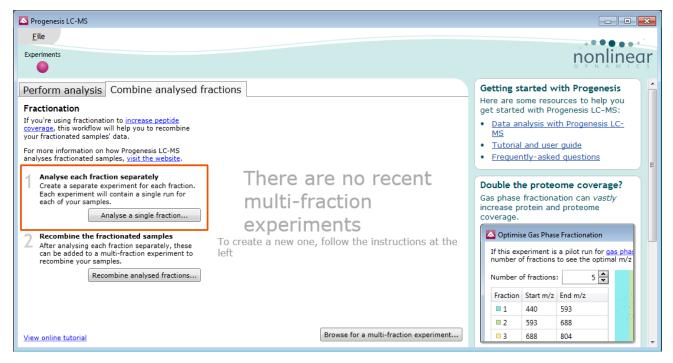
Score         Anova (r)           368         2.03E-06           403         3.89E-05           270         0.00443           574         0.00015           6 (Clostridium difficile)         0.0015	a (p ^ 4 06 V 05 V 43 V 151 V	# Score 69 63.9 132 103 147 101	10 1	Mass /	Mass error (p	RT (mins)	Charge	Tags -	<ul> <li>Abundance</li> </ul>	Conflicts	Create
368 2.03E-06 403 3.89E-05 270 0.00043 270 0.00015 4 46-00 [Clostridium difficile]	06 V 05 V 43 V 151 V	69 63.9 132 103	10 1				Charge	Tags	<ul> <li>Abundance</li> </ul>	Conflicts	Deptide (
403 3.89E-05 270 0.00443 574 0.00015 (Clostridium difficile)	05 🔽 43 🔽 151 🗹	132 103		1669.889	0.0302						replice:
270 0.00443 574 0.00015 [Clostridium difficile]	43 🔽		10 1		010002	42.1	3 .		4.75E+06	0	🕥 IAD
574 0.00015 4.445-02 0.005 or [Clostridium difficile]	151 🔽	147 101		1669.888	0.646	42.1	2		4.38E+06	0	S IAD
Clostridium difficile]			10 1	1230.609	0.44	22.7	2 .	$\sim$	3.07E+06	0	S AAD
[Clostridium difficile]		166 125	10 2	2317.115	0.168	38.7	2 .	$\sim$	5.55E+06	0	🕥 LES
	1		9 2	2317.115	0.201	38.7	3 .	/X	3.09E+06	0	🌖 LES
llin subunit [Clostridium		] 8 107	10 1	1716.857	0.429	30.4	2 .	/X	1.73E+06	0	🌖 VNT
365 0.0132	2 🗸	564 51.2	4 1	1716.858	0.394	30.4	3 .	$\mathbf{Z}$	3.57E+05	0	🌍 vnt
286 7 74F-04	06 T	283 49 5	10 1	1676 838	1 37	34.5	3.		7 59F+05	n	வ тпп
ein Score Peptid		es of conflic	Hits Mas		s error (p RT	(mins) C	harge Tag	•	Abundance (	Conflicts Pe	antida Saci
					III						
		▶ ◀	▶ ≺	× [	۶ - ۲	۲	۰ ( m	•	► < []	▶ <	

**Note**: the flagellin subunit has **no unique** peptides (brackets after the peptides field in the Proteins table as shown above) as they are all present in flagellin protein hence the reason for grouping. As a result all the conflicts are internal to the group.

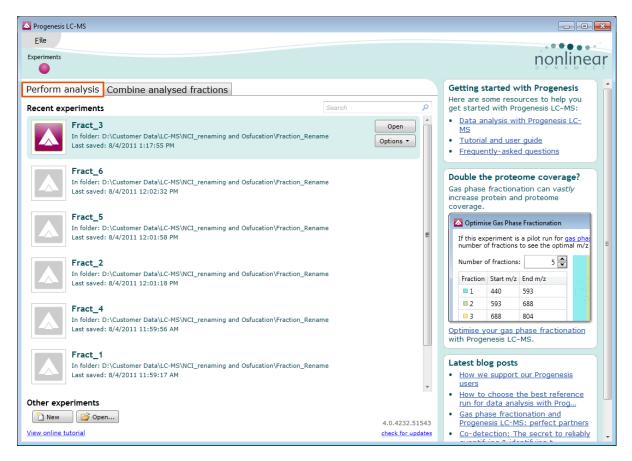
Before recombining the fractions make sure all the single fraction experiments are complete.

## **Completion of Fractionation Workflow Step 1**

The first stage in the analysis of a Fractionated experiment is completed when you have analysed all the single-fraction experiments, in this example there are 6 fractions, therefore 6 experiments. For each experiment this includes the identification of proteins and resolution of any peptide conflicts as described in the previous sections.



The six experiments will appear in the Perform Analysis tab and can be accessed individually.



The next section describes the workflow involved in the 'Combining' of these single fraction experiments to generate a 'multi-fraction' experiment.

# Fractionation Workflow Step 2

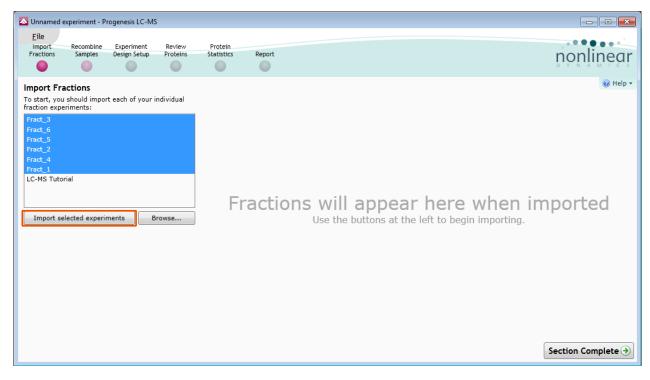
The combining of the single fraction experiments is performed in the second stage of the Fractionation workflow.

#### Select Recombine analysed fractions...

Progenesis LC-MS		
<u>F</u> ile		
Experiments		ponlinear
Perform analysis Combine analysed frac	tions	Getting started with Progenesis
Fractionation		Here are some resources to help you get started with Progenesis LC-MS:
If you're using fractionation to increase peptide coverage, this workflow will help you to recombine your fractionated samples' data.		Data analysis with Progenesis LC- <u>MS</u>
For more information on how Progenesis LC-MS analyses fractionated samples, visit the website.		<u>Tutorial and user guide</u> <u>Frequently-asked questions</u>
Analyse each fraction separately Create a separate experiment for each fraction.	There are no recent	Double the proteome coverage?
Each experiment will contain a single run for each of your samples.	multi-fraction	Gas phase fractionation can <i>vastly</i> increase protein and proteome
Analyse a single fraction	experiments	coverage.
Alter analysing each fraction separately, these	To create a new one, follow the instructions at the left	Optimise Gas Phase Fractionation
recombine your samples.		number of fractions to see the optimal m/z
Recombine analysed fractions		Number of fractions: 5
		Fraction Start m/z End m/z
		2 593 688
<u>View online tutorial</u>	Browse for a multi-fraction experiment	3 688 804

# **Stage 1 Import Fractions**

The Import Fractions stage of the work opens, select the experiments that correspond to the (6) fractions.

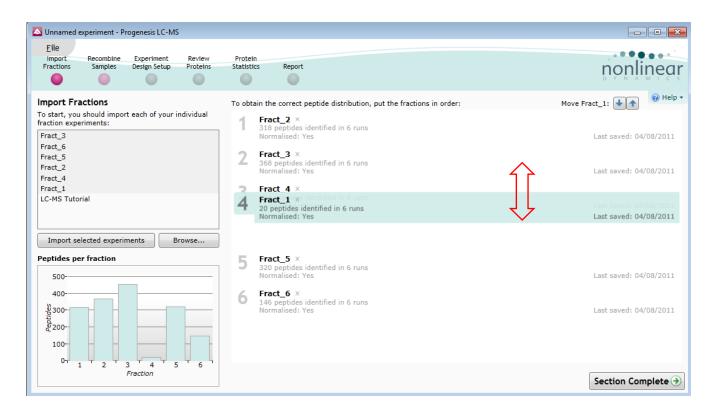


#### Click Import selected experiments

Details for each single fraction experiments appear in the panel showing numbers of identified peptides.

Unnamed experiment - Progenesis LC-MS		
Eile Import Recombine Experiment Review Fractions Samples Design Setup Proteins	Protein Statistics Report	nonlinear
Import Fractions	To obtain the correct peptide distribution, put the fractions in order:	Move Fract_4:
To start, you should import each of your individual fraction experiments: Fract_3 Fract_6 Fract_5 Fract_2 Fract_2 Fract_4 Fract_1 LC-MS Tutorial Import selected experiments Browse	<ol> <li>Fract. 3 × 368 peptides identified in 6 runs Normalised: Yes     </li> <li>Fract_6 × 146 peptides identified in 6 runs Normalised: Yes     </li> <li>Fract_5 × 320 peptides identified in 6 runs Normalised: Yes     </li> <li>Fract_2 × 318 peptides identified in 6 runs Normalised: Yes     </li> </ol>	Last saved: 04/08/2011 Last saved: 04/08/2011 Last saved: 04/08/2011 Last saved: 04/08/2011
Peptides per fraction 500-	5 Fract_4 × 454 peptides identified in 6 runs Normalised: Yes	Last saved: 04/08/2011
	6 Fract_1 × 20 peptides identified in 6 runs Normalised: Yes	Last saved: 04/08/2011
Fraction		Section Complete 🌖

You can adjust the order of the single fraction experiments to reflect the order of the fractions by dragging the single fraction experiments to the correct position.



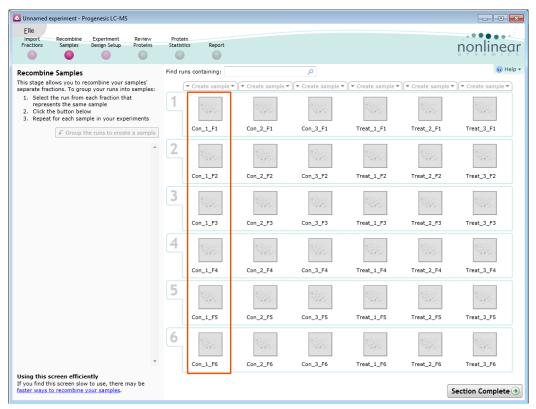
Note: the graph of 'peptides per fraction' updates to reflect the new order.

To move to the Recombine Samples stage click Section Complete.

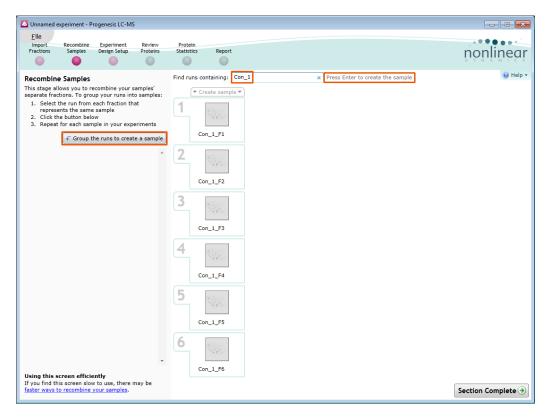
# **Stage 2 Recombine Samples**

At this stage you will recombine the samples by selecting the runs that correspond to each sample from the single fraction experiments.

**Note**: how efficiently you use this page will depend on how methodically you have named the various sample runs. For this example Samples are Con\_1, 2 and 3 and Treat\_1, 2 and 3



Typing **Con\_1** in the **Find runs containing** search box will locate the runs corresponding to sample Con\_1.



Click 'Enter' or Group the runs to create a sample in the left hand panel

Note: using Enter will set the sample name as Con\_1, overtype to rename as required.

Unnamed experiment - Progenesis LC-MS							
Fractions Recombine Experiment Review Proteins	Prote Statist						nonlinear
Recombine Samples This stage allows you to recombine your samples' separate fractions. To group your runs into samples:	Find ru	rns containing:	Create sample *	♀ ▼ Create sample ▼	Create sample *	▼ ) (▼ Create sample ▼	Ø Help ▼
<ol> <li>Select the run from each fraction that represents the same sample</li> <li>Click the button below</li> <li>Repeat for each sample in your experiments</li> </ol>	1						
✓ Group the runs to create a sample		Con_2_F1	Con_3_F1	Treat_1_F1	Treat_2_F1	Treat_3_F1	
Con_1 × *	2						
* Con_1_F3 * Con_1_F4		Con_2_F2	Con_3_F2	Treat_1_F2	Treat_2_F2	Treat_3_F2	
* Con_1_F5 * Con_1_F6	3						
		Con_2_F3	Con_3_F3	Treat_1_F3	Treat_2_F3	Treat_3_F3	
	4						
		Con_2_F4	Con_3_F4	Treat_1_F4	Treat_2_F4	Treat_3_F4	
	5						
		Con_2_F5	Con_3_F5	Treat_1_F5	Treat_2_F5	Treat_3_F5	
	6				E.	S.C.	
Using this screen efficiently		Con_2_F6	Con_3_F6	Treat_1_F6	Treat_2_F6	Treat_3_F6	
If you find this screen slow to use, there may be faster ways to recombine your samples.						2	iection Complete 🏵

Repeat for the remaining samples.

🔼 Unnamed e	kperiment - P	rogenesis LC-M	;			
Eile Import Fractions	Recombine Samples	Experiment Design Setup	Review Proteins	Protein Statistics	Report	nonlinear
separate fract 1. Select t represe 2. Click th	ows you to re tions. To grou he run from nts the same e button belo for each sam		o samples: at eriments			🧭 Heip +
Con_3 • Con_3_ • Con_3_ • Con_3_ • Con_3_ • Con_3_ • Con_3_	F2 F3 F4 F5		× ×			
Treat_1      Treat_1,     Treat	_F2 _F3 _F4 _F5		×	A	l rur	The section Complete button to continue analysis.
Treat_2 • Treat_2, • Treat_2, • Treat_2, • Treat_2, • Treat_2, • Treat_2, • Treat_2, • Treat_2,	_F2 _F3 _F4 _F5		×			
Treat_3 * Treat_3, * Treat_3	_F2 _F3 _F4 _F5		×			
	s screen slov	ently v to use, there i your samples.	may be			Section Complete 🕥

**Note**: as mentioned before other ways of Recombining the samples can be applied, depending on the naming conventions used; use the link, bottom left, to see the alternatives.

Having completed the recombination of the samples, click Section Complete.

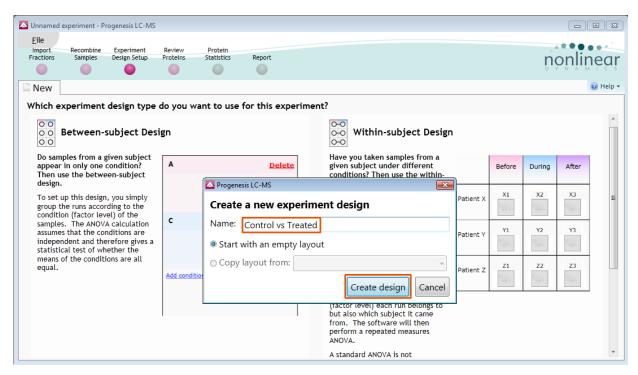
Note: at this point the data is re-normalised to account for the fractionation of the samples.

# Stage 3 Experiment Design Setup

Having recombined your samples you can now define the experimental designs most appropriate for your original experiment.

This experiment contains 2 conditions: Control and Treated and uses the **Between-subject design** to group the analysed samples to reflect the Biological conditions in the original study.

Select **Between-subject** and give design an appropriate name.



Highlight the samples, to add them on to a new condition click on Add Selected Samples to Condition

Elle   imports   Samples   Design Setup   Proteins   Statistics   Report	Unnamed experiment - Progenesis LC-MS					
Control vs Treated I × New Setup conditions Setup the conditions that you want to compare below (e.g., control, drug A, etc), and then assign each of your samples to the correct condition Control Delette Con_1 Remove Con_2 Remove Con_3 Remove	Import Recombine Experiment Review		Report			nonlinger
Setup conditions       Samples       Add Selected Samples to Condition       Search         Setup the conditions that you want to compare below (e.g., control, drug, a, etc.), and then assign each of your samples to the correct condition.       Samples       Add Selected Samples to Condition       Search         Control       Delete Con_1 Remove Con_2 Remove Con_3 Remove       Control       Treat_3						
Setup the conditions that you want to compare below (e.g., control, drug A, etc), and then assign each of your samples to the correct condition. Control Delete Con_1 Remove Con_2 Remove Con_3 Remove	Control vs Treated I × New					🕜 Help 🔻
Control Deleter Con_1 Remove Con_2 Remove Con_3 Remove	Setup the conditions that you want to compare below (e.g., control, drug A, etc), and then assign each of	Samples				
Con_1 Remove     Image: Con_2 Remove       Con_2 Remove     Image: Con_3 Remove	Control Delete		Control	Big.	Bill.	
Con_3 Remove	Con_1 Remove					
	Con_2 Remove					
Add condition						
Section Complete 🤿	Add condition					

Click Section Complete to move to Protein View

# **Stage 4 Review Proteins**

The recombined data can now be viewed at the level of the Proteins.

File Import Recor Fractions Sam			Protein Statistics	Repor	t					nonlinea
No filter appli	Create	Search		Q						Protein options
Accession	Peptide count	Confidence score	Anova (p)	Tag 💌	Max fold change	Fractions	Occurrences	Highest Mean	Lowest Mean	
🄰 gi 20629315	1	51.4	0.000344		3.72	123456	1	Control	Treated	50S ribosomal protein L7/L12 [Clostridium diff
🌒 gi 85279330	1	52.4	0.00844		2.16	123456	2	Control	Treated	PTS system, IIB component [Clostridium diffici
🄰 gi 72029336	1	78.3	0.00847		100	123456	1	Treated	Control	flagellin [Clostridium difficile]
🍯 gi 64841839	4	240	0.00901		2.06	123456		Control	Treated	enolase [Clostridium difficile 630]
🍯 gi 65076322	1	55	0.0126		2.27	123456	1	Treated	Control	transketolase [Clostridium difficile 630]
🍯 gi 46211184	2	117	0.0146		1.71	123456	2	Treated	Control	50S ribosomal protein L2 [Clostridium difficile
🔰 gi 42170149	1	45.8	0.0148		3.81	123456	1	Treated	Control	translation elongation factor G [Clostridium dif
🗿 gi 17787717	2	129	0.0149		3.59	123456	2	Treated	Control	30S ribosomal protein S4 [Clostridium difficile
gi 20629287	2	82.9	0.0161		1.83	123456	1	Treated	Control	tellurium resistance protein [Clostridium difficil
🄰 gi 51703916	4	263	0.0165		1.77	123456	3	Treated	Control	D-alaninepoly(phosphoribitol) ligase subunit
gi 04388227	1	41.7	0.0168		1.51	123456	1	Control	Treated	2-hydroxyisocaproate-CoA transferase [Clostri
🍯 gi 63129634	1	48.6	0.0177		1.79	123456	1	Control	Treated	cell surface protein [Clostridium difficile QCD-6
		x					•	÷ · ·		
Selected prot		e [Clostridiu		ile 630]						
: 15.0 -			Control						Trea	ited
L15.0			1 1							- 

And at the peptide level when you click view peptide measurements

A 1	Innam														
1	<u>F</u> ile Import	Re	combine i	enesis LC-MS											••••
F	raction	15 5	amples D	Design Setup Prot	teins Statis		t							noi	nlinea
_	Back			olase [Clostri	idium diffi	cile 630]									
		n: 🌚 giļ	64841839												
ept E	tides #	Score	Anova (n)	Max Fold Change	Highest Mean	Lowest Mean	Abundance	m/7	Charge	Retention Time	Eraction		Macs error (nom)	Peptide Sequence	Modificatio
	# 1135			1.2	Control	Treated		508.2809	-	23.265	1 2 3		1.47	GEMVHVNDR	Houncatio
	1388			1.11	Control	Treated		616.8116		28.624		4 5 6	0.09	DWGEECQAQYK	
	431		0.0888	4.03	Control	Treated	7.158E+05	602.8294		29.958		4 5 6	22.26	FVNNYYESEMK	
	666	55.17		1.4	Control	Treated	2.37E+05	508.2808		23.417			1.15	GEMVHVNDR	
	772		0.462	1.2	Control	Treated		554.7858		23.052		4 5 6	0.89	NFDFLDYGIR	
					, 										
						Sta	ndardised		ion Pro	ofiles	•				
			Contra			Sta	ndardised			o <b>files</b> ated	•				
Standardised Normalised Abundance	20 15 10 00 -05		Contro	ol o		Sta	ndardised				•				

Click back to return to the Protein View

Using the Protein Tags you can generate a list of proteins based on similar properties and thresholds.

Right click on a protein in the table and use the Quick Tags to generate tags for proteins with **Anova p-value≤0.05** and a **Max fold change≥2**.

Eile Import Recor Fractions Sam	ples Design Se		Protein Statistics	Repo	rt )					
No filter appli	Create	Search		Q						Protein options
ccession	Peptide count	Confidence score	Anova (p)	Tag 👻	Max fold change	Fractions	Occurrences	Highest Mean	Lowest Mean	Description
gi 20629315	1	51.4	0.000344		3.72	123456	1	Control	Treated	50S ribosomal protein L7/L12 [Clost
🔰 gi 85279330	1	52.4	0.00844		2.16	123456	2	Control	Treated	PTS system, IIB component [Clostri
) gi 72029336	1	78.3	0.00847		100	123456	1	Treated	Control	flagellin [Clostridium difficile]
) gi 64841839	No tags to assig	n	0.00901		2.06	123456		Control	Treated	enolase [Clostridium difficile 630]
gi 65076322	New tag		0.0126		2.27	123456	1	Treated	Control	transketolase [Clostridium difficile 6
) gi 4621118	Quick Tags	Ano	va p-value	_	1.71	123456	2	Treated	Control	50S ribosomal protein L2 [Clostridiu
) gi 42170149	Edit tags		fold change		3.81	123456	1	Treated	Control	translation elongation factor G [Clos
) gi 1778771)	2		Jence		3.59	123456	2	Treated	Control	30S ribosomal protein S4 [Clostridiu
gi 20629287	2	82.9 Mod	dification		1.83	123456	1	Treated	Control	tellurium resistance protein [Clostrie
gi 51703916	4	263	0.0165		1.77	123456	3	Treated	Control	D-alaninepoly(phosphoribitol) liga
gi 04388227	1	41.7	0.0168		1.51	123456	1	Control	Treated	2-hydroxyisocaproate-CoA transfera
gi 63129634	1	48.6	0.0177		1.79	123456	1	Control	Treated	cell surface protein [Clostridium diff
10.00000					050		-		- · ·	the second second
Selected prot	ein: enolas rements			ile 630	]				Treated	
Arcsimh Normalised Abur 14.5			<u><u>×</u></u>							

Using the 'Tag' filters the list is reduced to the relevant proteins. Details of these proteins can be exported by selecting **Export Protein Measurements** from the **File** menu.

	npline Experime Iples Design Se		Protein Statistic		t					nonlinea
<b>Tag filter ap</b> proteins may		it Search			٩					Protein options @ Help
Accession	Peptide count	Confidence score	Anova (	o) Tag 👻	Max fold change	Fractions	Occurrences	Highest Mean	Lowest Mean	Description
gi 20629315	1	51.4	0.00034	4 🍈	3.72	123456	1	Control	Treated	50S ribosomal protein L7/L12 [Close
gi 85279330	1	52.4	0.00844	۷	2.16	123456	2	Control	Treated	PTS system, IIB component [Clost
🍯 gi 72029336	1	78.3	0.00847	۷	100	123456	1	Treated	Control	flagellin [Clostridium difficile]
🄰 gi 64841839	4	240	0.00901	4	2.06	123456	] 3	Control	Treated	enolase [Clostridium difficile 630]
) gi 65076322	1	55	0.	ilter the prote	ins				_	sketolase [Clostridium difficile
) gi 42170149	1	45.8	0.0							slation elongation factor G [Clo
gi 17787717	2	129	0.1	eate a filte	er oteins based on a	selection of their	tage Move tag	to the appropr	riate hoves to	ribosomal protein S4 [Clostrid
gi 04526507	1	47.7			For more guidance				Indie Boxes to	preductase-family protein [Clos
🗿 gi 64041498	4	262	0.0 Av	ailable tags:			Show proteins	that have all of	these tans:	redoxin [Clostridium difficile 63
gi 99669795	1	40.6	0.0					nange ≥ 2 (262		hylate kinase [Clostridium diffic
🍯 gi 64841853	2	99.9	0.0				1	lue ≤ 0.05 (37		ellin [Clostridium difficile]
gi 65076352	1	77.1	0.0				Show proteins	that have at lea	ast one of these	e redoxin [Clostridium difficile 63
100004044	-		~				tags:			
										,
Selected pro		e [Clostridiu	m							
							Hide proteins t	hat have any of	these tags:	_
; 15.0 -		C	Cont							
Abu										
<b>Pes</b> 14.5			-							
- June			C	lear the filter				OK	Cancel	
ž <u>F</u> 14.0										
L15.0									1	
< −									· · · ·	

Details of these proteins can be exported by selecting Export Protein Measurements from the File menu

Δ υ	nnamed experimer	nt - Progenesis L	C-MS							
Ē	ile									
	Save			Review Proteins	Protein Statistics					
	Close									
	Export Protein Measurements									
	Import Protein Accessions as Tag Search									
	Export Peptide Me	easurements								
	Import Additional	Protein Data		ence score	Anova (p)					
					0.000344					
×	Exit	_		1	0.00844					
<b>S</b>	🔮 gi 72029336 1 78.3									
S 🔇	ji 64841839	4	240		0.00901					
S 📀	ji 65076322	1	55		0.0126					

Choose properties to be include	ded in exported file
Accession	
Peptide count	
Peptides used for quantitation	ation
Confidence score	
📝 Anova (p)	
Max fold change	
Fractions	
Occurrences	
Highest mean condition	
Lowest mean condition	
Description	
Normalized abundance	
Tags	

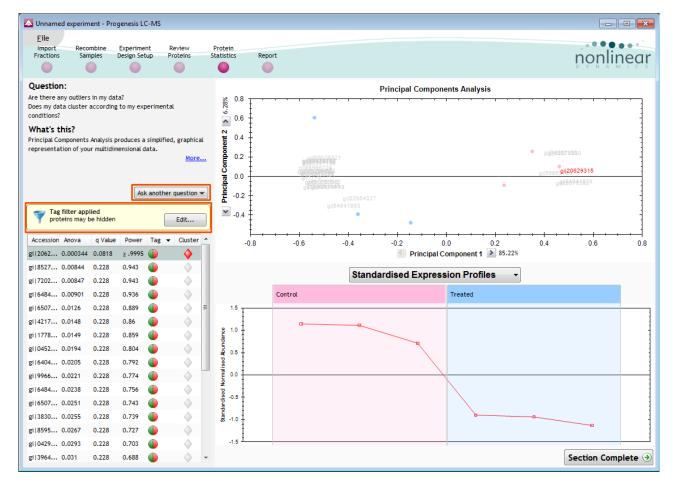
A dialog allows you to control the details of the output file.

Now move to the Protein Statistics section by clicking on **Protein Statistics** icon on the workflow at the top of the screen.

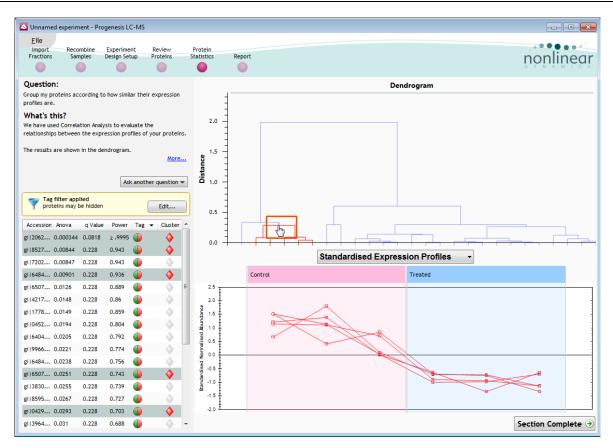
Finally your recombined data can be saved as a Multi-fraction experiment.

#### **Stage 5: Protein Statistics**

Protein Statistics opens with a Principal Components Analysis (PCA) for all the proteins displayed.



The Multivariate Stats can now be applied to all or subsets of proteins as determined by the current Tag filters. Allowing you to identify similar paterns of expression using the Correlation Analysis.



Now move to the Report section to report on Proteins and /or peptides.

#### **Stage 6: Reporting**

The **Report Design** stage allows you to select what views you want to include in a report based on the list of **currently selected proteins.** 

As an example we will create a report for **only** the proteins with **Anova p-value≤0.05** and a **Max fold change≥2.** 

Filter the proteins	
Create a filter Show or hide proteins based on a selection of the create the filter. For more guidance, please see th	
Available tags:	Show proteins that have all of these tags:
	Max fold change ≥ 2 (262 proteins)
	Anova p-value ≤ 0.05 (37 proteins)
	Show proteins that have at least one of these tags: Hide proteins that have any of these tags:
Clear the filter	OK Cancel

- 1. First reduce the proteins to report on by selecting the tags: Anova p-value≤0.05 and a Max fold change≥2.
- 2. Expand the various Report Design options (by default they are all selected)
- 3. Un-tick as shown below

#### 4. Click Create Report

File Import ractions	Recombine Samples	Experiment Design Setup	Review Protein			nonline
oteins 😎 Tag filt	er applied					Report Design
Y features	may be hidd	len		Edit		Title Significantly Changing Proteins
ccession i 20629315		Fold 3.7	Tag 🗸	Description 505 ribosomal protein L7/L12 [Clostridium difficile 630]	*	Select the sections you wish to include in your report: $$
i 85279330 i 72029336 i 64841839	0.00847	2.2 100.4 2.1	•	PTS system, IIB component [Clostridium difficile 630] flagellin [Clostridium difficile] enolase [Clostridium difficile 630]		
i 65076322 i 42170149		2.3 3.8	•	transketolase [Clostridium difficile 630] translation elongation factor G [Clostridium difficile 630]		✓ Protein table ✓ Peptide tables
i 17787717 i 04526507 i 64041498	0.0194	3.6 858.0 3.3	•	30S ribosomal protein S4 [Clostridium difficile 630] nitroreductase-family protein [Clostridium difficile 630] thioredoxin [Clostridium difficile 630]		
99669795  64841853	0.0221	15.3 2.2	•	adenylate kinase [Clostridium difficile 630] flagellin [Clostridium difficile]	E	
65076352  38304314	0.0255	4.8 3.9	•	thioredoxin [Clostridium difficile 630] (R)-2-hydroxyisocaproate dehydrogenase [Clostridium difficile]		Create Report
85956724 04292138	0.0293	2.6 2.5 8.3		cell surface protein [Clostridium difficile 630] flagellin [Clostridium difficile] pvruvate-flavodoxin oxidoreductase [Clostridium difficile OCD-23m63]		
39642159  48078745		2.1	•	aspartate aminotransferase [Clostridium difficile 630]		
00347190  82751345		2.6 2.8	•	cell surface protein [Clostridium difficile 630] PTS system, IIB component [Clostridium difficile 630]		
02954327		2.9 3.4	•	cell surface protein [Clostridium difficile 630] ferredoxin-NADP(+) reductase subunit alpha [Clostridium difficile 630]	1	
21210623	0.046	3.4		transcription elongation factor [Clostridium difficile 630]	*	

This opens a dialog to allow you to save the report, after which it will be opened in the form of a web page.

Signific	Significantly Changing Proteins											
Experiment	: Unnam	ed exp	eriment									
Report crea	ated: 05/	08/201	1 15:17:1	4								
Proteins												
Protein building options Protein grouping <b>Group similar proteins</b> Protein quantitation <b>Using only features with no protein conflicts</b>												
Accession	Peptides	<u> </u>	Anova (p) *	Fold	Tags	Fractions	Description	Average Nor Abundances	malised			
								Control	Treated			
gi 64041498	4	261.88	0.02	3.31		1 2 3 4 5 6	thioredoxin [Clostridium difficile 630]	2.34e+005	7.73e+005			
<u>gi 64841839</u>	4	239.74	9.01e-003	2.06		123456	enolase [Clostridium difficile 630]	1.27e+006	6.14e+005			
gi 38304314	2	133.82	0.03	3.90		123456	(R)-2-hydroxyisocaproate dehydrogenase [Clostridium difficile]	5.53e+004	2.16e+005			
gi 17787717	2	128.88	0.01	3.59		1 2 3 4 5 6	305 ribosomal protein 54 [Clostridium difficile 630]	8.94e+004	3.21e+005			
gi 64841853	2	99.94	0.02	2.22		1 2 3 4 5 6	flagellin [Clostridium difficile]	1.16e+005	2.58e+005			
<u>gi 05386167</u>	2	95.22	0.05	2.54		123456	small acid-soluble spore protein A [Clostridium difficile 630]	9.35e+004	2.37e+005			
<u>gi 48078745</u>	2	94.59	0.03	2.11		123456	aspartate aminotransferase [Clostridium difficile 630]	2.71e+005	5.72e+005			
<u>gi 85956724</u>	1	82.04	0.03	2.59		123456	cell surface protein [Clostridium difficile 630]	1.20e+005	3.11e+005			
gi172029336	1	78.27	8.47e-003	100.38		1 2 3 4 5 6	flagellin [Clostridium difficile]	2040.73	2.05e+005			

Click on the **Accession No**. in the proteins section of the Report and this will take you to the Assigned peptides for this protein

l peptides														
Sequence	Feature	Score	Hits	Mass	Charge	Fr	acti	on			Modifications	In quantitation	Average Nor Abundances	
													Control	Treated
DWGEECQAQYK	1388	67.65	2	1231.6086	2	1	2	3 4	4 5	6		yes	7.98e+004	7.18e+004
FVNNYYESEMK	431	52.04	1	1203.6443	2	1	2	3 4	4 5	6		yes	7.16e+005	1.78e+005
GEMVHVNDR	666	55.17	5	1014.5470	2	1	2	3 4	4 5	6		yes	2.37e+005	1.70e+005
GEMVHVNDR	1135	61.69	2	1014.5473	2	1	2	3 4	4 5	6		yes	7.38e+004	6.17e+004
VFDFLDYGIR	772	50.04												
	,,,,	58.36	1	1107.5570	2	1	2	3 4	1 5	6		yes	1.61e+005	1.33e+005
gi   3830431 R)-2-hydroxyisa 2 peptides Sequence	4	e deh	ydrog			m d	liffi		e]	6	Modificatio		Average N	łormalised
R)-2-hydroxyiso 2 peptides	<u>4</u> ocaproat	e deh	ydrog	genase [Clo	stridiu	m d	liffi	icile	e]	6	Modificatio	ns In	Average N	łormalised
R)-2-hydroxyiso 2 peptides	4 ocaproat	re deh	ydrog	genase [Clo	stridiu Char	m d	liffi	icile	e]	6		ns In	Average N Abundan	lormalised ces

Having closed the report it can be reopened by double clicking on the saved html file.

Note: you can also copy and paste all or selected sections of the report to Excel and/or Word.

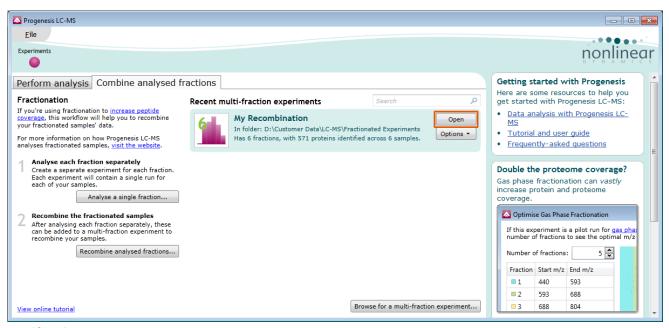
Note: there are separate panels for reporting on Proteins and Peptides

Finally your recombined data can be saved as a Multi-fraction experiment.

#### Stage 7: Saving a Multi-Fraction experiment

When you opened the Fractionation workflow and started working with the recombination of your 'Single Fraction Experiments' the workflow recognises the current experiment as '**Unnamed**' this status will change as you close and/or save the experiment.

On saving the new multi-fraction experiment appears on the Combine analysed fractions page



Details of the new experiments location (folder), number of fractions, samples and total identified proteins. **Note**: you can reopen the experiment by either double clicking on it or using **open**.

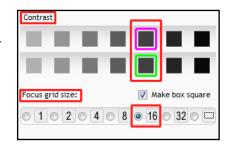
This completes a guided tour through using Progenesis LC-MS to analyse fractionated data.

# Appendix 1: Manual assistance of Alignment

#### Approach to alignment

To place manual alignment vectors on a run (A2 in this example):

- 1. Click on Run A2 in the **Runs** panel, this will be highlighted in green and the reference run (A1) will be highlighted in magenta.
- 2. You will need approximately 5 10 **alignment vectors** evenly distributed from top to bottom of the whole run.
- 3. First ensure that the size of the focus area is set to **8 or 16** in the Focus grid size on the bottom left of the screen.



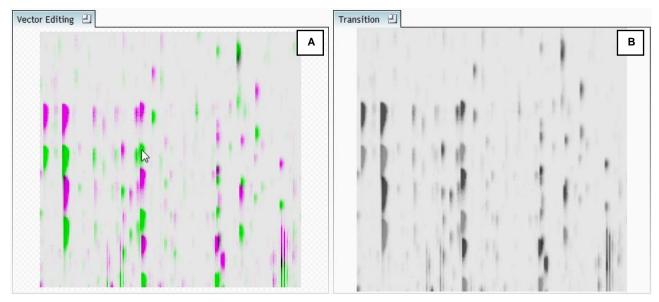
Click on an area (see below) in the **Whole Run** view (C) to refocus all the windows. Adjust Contrast as required.



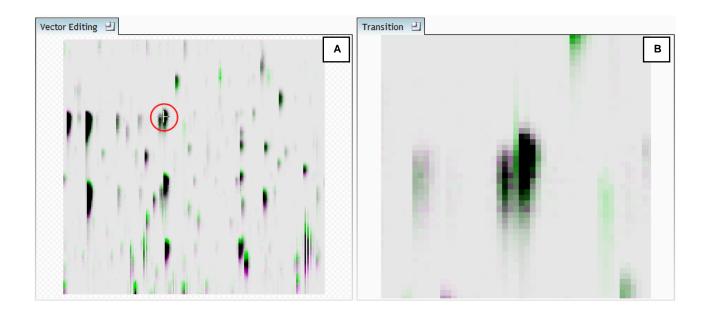
**Note**: the features moving back and forwards between the 2 runs in the **Transition** view indicating the misalignment of the two LC-MS runs

**Note**: The **Total Ion Chromatogram** view also reflects the misalignment of the 2 runs for the current Retention Time range (vertical dimension of the current Focus grid in the **Whole Run** view.

4. Click and hold on a green feature in Window A as shown below.



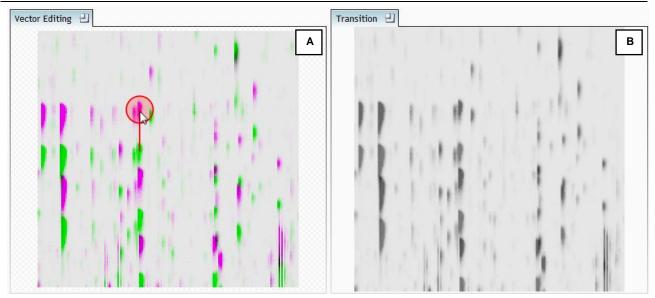
5. As you are holding down the left mouse button drag the green feature over the corresponding magenta feature of the reference run. The red circle will appear as shown below indicating that a positional lock has been found for the overlapping features.



Note: as you hold down the mouse button, window B zooms in to help with the alignment.

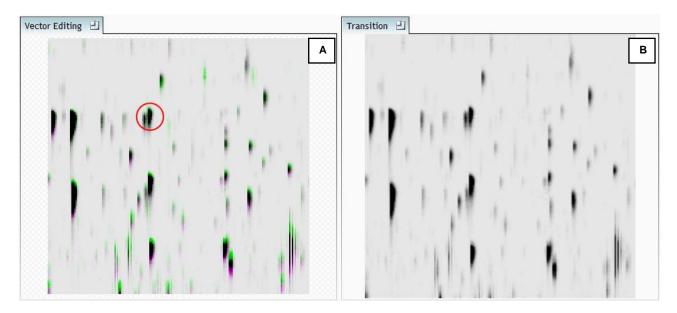
6. On releasing the left mouse button the view will 'bounce' back and a red vector, starting in the green feature and finishing in the magenta feature will appear.

#### Progenesis LC-MS Fractionation User Guide

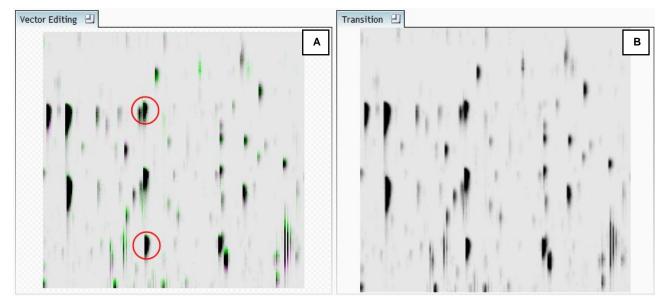


Note: an incorrectly placed vector is removed by right clicking on it in the Vector Editing window

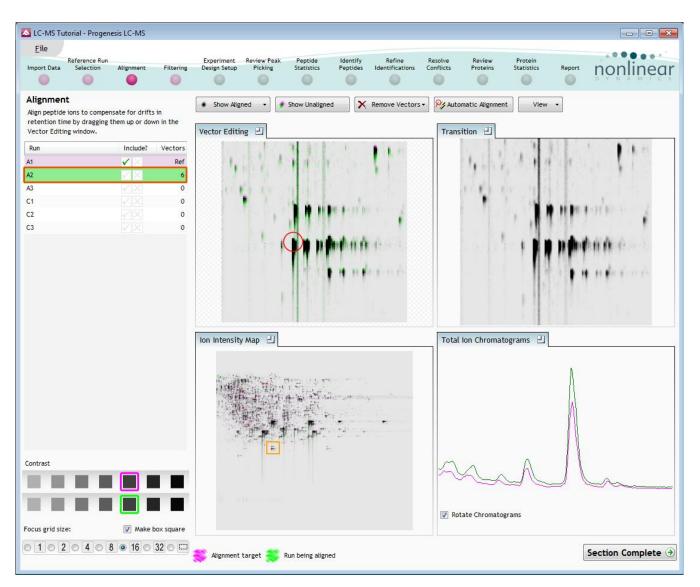
7. Now click **Show Aligned** on the top tool bar to see the effect of adding a single vector.



8. Additing an additional vector will improve the alignment further. **Note** this time as you click to add the vector it 'jumps' automatically to the correct position using the information from the existing alignment vector.



Repeat this process moving the focus from top to bottom on the Whole Run view

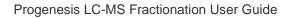


Note: the number of vectors you add is recorded in the Runs table

10. Repeat this process for all the runs to be aligned.

The number of manual vectors that you add at this stage is dependant on the misalignment between the current run and the Reference run. In many cases only using the Automatic vector wizard will achieve the alignment.

Also the 'ease' of addition of vectors is dependant on the actual differences between the LC-MS runs being aligned





11. Then select Automatic Alignment to bring up the Automatic Alignment dialog and click **OK**. The automatic alignment process will begin, using the manual vectors you have added to aid in automatic vector placement.

A	utomatic	lignment	×
	Select the	runs for automatic alignment vector generation	
Ι.	Add Ru	n Notes	Vectors
	🔳 A1	this run does not need to be aligned as it is the alignment reference	Ref
	A2	run has user vectors	6
	A3	run has user vectors	6
	🗹 C1	run has user vectors	6
	🔽 C2	run has user vectors	6
	🔽 C3	run has user vectors	6
		ок	Cancel

**Note**: the tick boxes next to the 'Run name control' which control whether vectors will be generated for each run.

To review the vectors, automatic and manual return to page 9

## **Appendix 2: Within-subject Design**

To create a **Within-subject Design** for your data set select this option on the **Experiment Design Setup** page and enter the name of the design.

In this example there are 3 Subjects (i.e. patients A, B and C) who have been individually sampled: Before(1), During (2) and After (3) treatment

When the design page opens use the **Add Subject** and **Add Condition** buttons to create the matrix that fits your experimental design, over typing the names as required.

Then Drag and drop the Samples on to the correct 'cell' of the matrix.

LC-MS Tutorial - Progenesis LC-MS						
Eile Reference Run Experimer	nt Review Peak Pe	ptide Identify	Refine Re	solve Review	Protein	
Import Data Selection Alignment Filtering Design Set	up Picking Sta	tistics Peptides	Identifications Cor	nflicts Proteins	Statistics Repo	nonlinear
						🔞 Help 🗸
Before During and After Treatment I	× 🗋 New	-				W Help +
Setup conditions and subjects						
Setup the conditions and subjects for your experiment design on the right, and then assign each		Before	During	After	Add Condition	
of your samples to the correct subject/condition cell in the grid.						
<ol> <li>Add a column for each condition.</li> <li>Add a row for each subject.</li> </ol>					1	
3. Drag each of your samples to the correct location	Patient A	A1	A2	A3		
in the grid.		Sel.	B.C.			
Filter samples: 🔎				1.00		
					1	
C2	Pateint B	B1	B2	B3		
		Sec.	and the second	Sec.		
			and the second sec			
C3					]	
28 S 1 1 2	Patient C	C1	elect Sample	Select Sample		
		Sec.	alect Sample	Select Sample		
	Add Subject					
						Section Complete 🏵

You can create additional Experimental Designs using the New tab

All of these Experimental Designs are available at all the following stages in the LC-MS workflow.